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13. ABSTRACT *(Maximum 200 words)*

The primary objective of this proposal is to elucidate the role of the phosphadityl inositol 3' kinase (PI-3' kinase) and its coupled downstream signaling pathways in the induction of mammary cancers. In particular, we have focussed on the activation of the PI-3' kinase signaling pathway and the Akt/PKB serine kinase which is one of the immediate downstream targets of activated PI-3' kinase. To this end, we have derived separate transgenic strains which express activated versions of either PI-3' or Akt kinase under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter. Preliminary phenotypic characterization of these strains revealed mammary epithelial specific expression of either activated PI-3' or Akt kinase resulted in the induction of low-grade mammary epithelial hyperplasias without the overt appearance of mammary tumors. To further explore the significance of activation of PKB/Akt and PI-3' kinase, we have interbred these separate strains of transgenic mice with transgenic mice expressing a mutant form of the polyomavirus (PyV) middle T (mT) antigen de-coupled from the PI-3' kinase. Although we are awaiting the results of the cross between the activated PI-3' kinase and mutant PyV mT strains, preliminary phenotypic characterization of bigenic mice co-expressing the mutant PyV mT oncogene has revealed that co-expression of Akt and mutant PyV mT oncogene resulted in dramatic acceleration in tumorigenesis.

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INTRODUCTION

The major focus of this Army funded research program is to assess the relative importance of the PI-3' kinase and its coupled signaling pathways in the induction of breast cancer. Recent studies have suggested that growth factor-mediated activation of the PI-3' kinase signaling pathway plays a critical role in mammary tumor progression. For example, we have demonstrated that mammary epithelial expression of a mutant PyV mT de-coupled from the PI-3' kinase signaling pathway results in a dramatic reduction in its capacity to induce metastatic mammary tumors (24). Previous studies with these mutant PyV mT strains revealed that the observed delay in tumor induction was a result of a dramatic elevation in apoptotic cell death observed in mammary epithelium derived from these strains (24). To directly assess the importance of PI-3' kinase coupled signaling pathways in mammary tumorigenesis, we initially sought to isolate separate strains of transgenic mice which express activated versions of both the PI-3' kinase (21) and its immediate downstream target, Akt/PKB in the mammary epithelium. Given the importance of these signaling pathways in providing cell survival signals (2, 4, 12, 13, 22-24), a second aim of this study was to assess whether co-expression of either activated forms of PI-3' kinase or Akt kinase could suppress the elevated levels of apoptotic epithelial cell death observed in these mutant PyV mT oncogene strains (24). To accomplish these goals we have generated several independent strains of transgenic mice that express either Akt or PI-3' kinase in the mammary epithelium. Preliminary phenotypic characterization of these transgenic strains has revealed that expression of either activated versions of Akt or PI-3' kinase results in the induction of mammary epithelial hyperplasias. Although mammary tumors have not yet arisen in the either the MMTV/activated PI-3' kinase or MMTV/activated Akt kinase, co-expression of the activated Akt with the mutant PyV mT oncogene in the mammary epithelium can completely rescue the defect in tumorigenesis observed in these mutant PyV mT strains. Interestingly, despite the rescue of tumorigenesis, bigenic mice co-expressing activated Akt and mutant PyV mT oncogene fail to exhibit the high rates of metastatic progression associated with expression of the wild type PyV mT oncogene. These observations suggest that activation of the Akt signaling pathway can compliment the induction of tumorigenesis but is unable recapitulate the metastatic properties of these PyV mT- induced tumors.

BODY

Generation and characterization of MMTV/activated Akt/PKB mice.

To derive transgenic mice that express an activated version of Akt containing aspartic acid substitutions at the two know phosphorylation sites (theronine 308 and serine 473) PKB (1) was introduced into am mouse mammary tumor virus expression cassette (20) (Fig 1 A). To facilitate the identification of transgene encoded protein an influenza-encoded haemiaglutnin (HA) tag was also inserted in-frame with the 5' portion of Akt coding sequences. After microinjection of one-cell mouse embryos with the MMTV/activated Akt construct a total of 11 independent transgenic founder lines were established. Of these 11 established transgenic lines, a total of 9 passed the transgene to their offspring (Table #1) To determine which of these transgenic strains were expressing in the mammary epithelium, RNase protection analyses was conducted on mammary tissue from these 9 lines with a riboprobe directed to the SV40 component of the transgene. The results revealed that only three of the 9 lines of MMTV/activated expressed significant levels of the activated Akt transgene (Table #1). Quantitative analyses of the RNase protection experiments

revealed that of the three expressing Akt strains, the Akt-7 and Akt-10 lines expressed the highest levels of transgene transcript in the mammary epithelium and thus were subjected to further molecular and pathological analyses. To confirm that transgene encoded transcript encoded Akt protein, we conducted immunoblot analyses on a variety of tissue from the Akt-7 and Akt-10 with antibodies directed to the HA tag. As observed with many other MMTV driven transgenes (9), the HA epitope tagged Akt protein was observed in the mammary tissue and male reproductive tract (Fig. 1C). Taken together, these observations suggest that we derived two independent transgenic strains that express elevated quantities of the activated Akt protein in the mammary epithelium.

To assess whether mammary-epithelial expression of activated Akt could perturb normal mammary gland development, we performed wholemount analyses on virgin mammary glands from the Akt-7 strain. The results revealed that in comparison to virgin nontransgenic mammary glands (Fig. 2A), the mammary glands derived from the Akt-7 strain exhibited mild ductal hyperplasia (Fig. 2B). Similar observations were made with the Akt-10 strain. Consistent with these observations, females from both the Akt-7 and Akt-10 strains are capable of functionally nursing their off spring suggesting that elevated expression of activated Akt does not impair normal mammary gland function. Female mice from both the Akt-7 and Akt-10 strains were also monitored for the development of palpable mammary tumors. Although female mice from both strains have been monitored for over year, we have not detected mammary tumors in these strains. These observations argue that activation of Akt is insufficient to induce mammary tumors.

Co-expression of activated Akt and a mutant PyV mT de-coupled from the PI-3' kinase in mammary epithelium can complement tumorigenesis but not metastasis.

Although our observations suggest that activation of Akt is not sufficient to induce mammary tumors, given its well characterized anti-apoptotic functions (12), mammary gland expression of Akt may be able to cooperate with other oncogenes to accelerate mammary gland tumorigenesis. One ideal system to test the importance of Akt in mammary tumorigenesis is the PyV MT oncogene. Mammary epithelial expression of PyV mT results in induction of mammary tumors in 100% of female carriers (14). More significantly, all tumor bearing transgenic mice eventually go on to develop metastatic mammary tumors (14). The potent transforming activity of PyV mT is due to capacity to associate and activate the Src family kinases, the PI-3' kinase and the Shc adapter protein (3, 5-8, 11, 17, 18). Consistent with this view, expression of mutant PyV mT antigen de-coupled from the PI-3' kinase or Shc signaling pathways exhibit a profound delay in tumor development (24). The impaired tumor development in transgenic mice de-coupled from PI-3' kinase signaling molecule is in turn correlated with high rates of apoptotic cell death (24). Given the fact that Akt is immediately downstream of activation of the PI-3' kinase pathway, mammary epithelial expression of activated Akt could conceivably complement the tumor defect observed in the PI-3'kinse defective PyV mT strains. To test this possibility, the MMTV/Akt-7 strain was interbred with a representative mutant PyV mT strain. (MTTV/MT315/322F strain) to generate bigenic mice co-expressing both activated Akt and mutant PyV mT de-coupled from the PI-3' kinase signaling molecule. Female mice carrying either mutant PyV mT, or activated Akt or both were then monitored for tumor development by physical palpation. The results of these analyses revealed that the bigenic mice (T50=46 days) developed mammary tumors with dramatically accelerated onset compared to parental mutant PyV mT strains (T50= 123 days) (Fig. 3). As expected the activated Akt strains alone failed to develop mammary tumors. Indeed, the onset of mammary tumors observed in mice co-expressing the mutant PyV mT oncogene and Akt was comparable to that seen

in transgenic mice expressing the wild-type PyV mT oncogene (14). However unlike the wild-type PyV mT strains which develop lung metastasis with 100% penetrance, only 30% of the mammary tumors from the bigenic mice metastasized to the lung. In fact, the rate of metastatic progression observed in these strains is comparable to the mutant PyV mT strains (24).

To determine if the enhanced rate of tumor onset observed in bigenic strains resulted in abnormal mammary gland development, we performed wholemount analyses on virgin mammary epithelium derived the mutant PI-3' kinase PyV mT transgenic mice and mice co-expressing activated Akt and mutant PyV mT (Fig. 2C and 2D). Consistent with the tumor kinetics, wholemount analyses of the bigenic mammary glands revealed that in contrast to parental PyV mT glands which exhibited global epithelial hyperplasias, the bigenic mammary glands were highly dysplastic with multiple neoplastic foci (Compare Fig. 2C and 2D). To confirm that enhanced tumor phenotype observed in the bigenic mice was due co-expression of activated Akt and the mutant PyV mT oncogene, we performed immunoblot analyses on tumor extracts derived from bigenic and mutant PyV mT tissues with antibodies specific to either the HA tag or PyV mT protein. As expected, the results of these analyses revealed that tissues derived from the bigenic tissues co-expressed both activated Akt and PyV mT proteins (Fig 4). Together, these observations suggest that activation of Akt can complement the observed defect in tumor development in mutant PyV mT strain but does recapitulate the metastatic phenotype characteristic of wild type PyV mT oncogene.

Isolation of mice expressing an activated form of the PI-3 kinase in the mammary epithelium of transgenic mice.

Another important goal of our Army sponsored research program was to derive transgenic mice that express a constitutively activated form of the PI-3' kinase in the mammary epithelium. To accomplish this, a cDNA encoding a activated form of catalytic subunit the PI-3' kinase containing a CAAX membrane localization motif (p110-CAAX) (19, 21) was inserted in MMTV expression cassette (Fig. 1B). To facilitate analyses of transgene expression, a c-myc epitope was placed in frame with the N-terminus of PI-3' kinase. To derive transgenic mice one cell mouse embryos were microinjected with this MMTV/PI-3' kinase fusion construct. Although the results are preliminary, we have derived 3 separate strains that have passed the transgene to their progeny (Table #1). In addition, we are currently deriving further strains. Future crosses of these activated PI-3' kinase strains with the mutant PyV mT strains de-coupled from the PI-3' kinase should allow us to elucidate their role in PyV mT mediated tumorigenesis and metastasis.

RESEARCH ACCOMPLISHMENTS

1. **Derivation and characterization of MMTV/activated Akt strains.**

2. **Interbreeding of MMTV/activated Akt strains with mutant PyV mT strains de-coupled from the PI-3' kinase.**

3. **Demonstration that expression of activated Akt can complement mammary tumorigenesis defect but not metastatic progression in mutant PyV mT de-coupled from the PyV mT oncogene.**

4. **Derivation of MMTV/activated PI-3' kinase strains.**

REPORTABLE OUTCOMES

1. Derivation of transgenic mice expressing activated versions of either activated Akt or PI-3' kinase in the mammary epithelium.

CONCLUSIONS

The results of our first year of funding from the US army have already provided important insight into role the PI-3' kinase and the Akt signaling molecules play in PyV mT mediated tumorigenesis and metastasis. We have demonstrated that activation of Akt serine kinase is not sufficient to induce mammary tumors. Given the expression of Akt in human breast cancer derived cell lines (15), these observations suggest that activation of Akt requires the concerted activation of other sets of oncogenes. Consistent with this hypothesis, we have further demonstrated that co-expression of the activated version of Akt can compliment the tumorigenesis defect in transgenic mice expressing a mutant form of PyV mT oncogene de-coupled from the PI-3' kinase signaling molecule (Fig 2, Fig. 3 and Fig. 4). However co-expression of activated Akt with the mutant PyV mT could not recapitulate the potent metastatic phenotype observed in the wild type PyV mT strains (14). These observations argue that activation of Akt functions primarily to promote tumor induction and have little influence on subsequent metastatic progression. The observation that Akt can compliment tumorigenesis but has little effect metastasis suggest activation of PI-3' kinase signaling pathway by PyV mT may have other distinct downstream targets. Indeed it has been demonstrated that phosphinotides 3' lipids generated by activation of the PI-3' kinase are capable of activating a number of other downstream molecules. For example, members of the Rac small GTP binding proteins and integrin linked kinase (ILK) are activated by these PI-3' kinase products (10, 21). Indeed, activation of either ILK or Rac/Rho family members have also been implicated in modulating cell motility and invasiveness (10, 16, 21). Future studies with these tumor models should allow us to assess the relative importance of these signaling molecules in modulating invasive phenotype.

A second important goal of our studies was to generate transgenic mice which express a constitutively activated version of the PI-3' kinase in the mammary epithelium. Although we are in process of generating and characterizing these strains, preliminary wholemount analyses of one of the activated PI-3' kinase strains has revealed that these mice develop extensive mammary epithelial hyperplasias. Our future focus will be to derive further transgenic lines to confirm this preliminary phenotypic observation. In addition, we plan to correlate any observed mammary phenotype with expression of the activated PI-3' kinase transgene. Ultimately, we plan to interbreed selected activated PI-3' kinase strains with the mutant PyV mT strain defective in PI-3' kinase signaling. Comparison of the mammary phenotypes from these crosses with the bigenic mice co-expressing activated Akt/mutant PyV MT will provide important insight into the relative contribution of these signaling molecules to the metastasis and tumorigenesis.

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APPENDICES

**CONTAIN UNPUBLISHED DATA*

Table 1. Transgene Expression in MMTV/Akt and MMTV/PI3K mice[a]

Line	Expression of transgene[b] in:												
	Female										Male		
	MGl	Brain	Heart	Kidney	Liver	Lung	Ovary	Salivary	Spleen	Thymus	Epidid	SemVes	Testes
Akt1	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt2	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt3	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt4	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt5[c]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt6	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt7	++	-	-	-	-	-	-	-	-	-	++++	++	-
Akt8	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt9	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt10	+	-	-	-	-	-	-	-	-	-	+++	++++	-
Akt11[c]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
PI3K1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
PI3K2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
PI3K3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[a] Expression of the Akt transgene in the mammary gland was initially determined via RNase protection analysis on 20 μg of total RNA with a probe directed against the SV40 polyA region of the transgene. Subsequently, expression of the Akt transgene in the mammary gland and other tissues of expressors was determined by Western blot analysis using the HA-11 monoclonal antibody (Babco) on 250 μg of total protein lysate pre-cleared in ProteinG-Sepharose.

[b] Relative levels of transgene expression: nd, no data; -, not detected; +, low; ++, intermediate; +++, high; ++++, very high; MGl, normal mammary gland; Epidid, epididymus; SemVes, seminal vesicles.

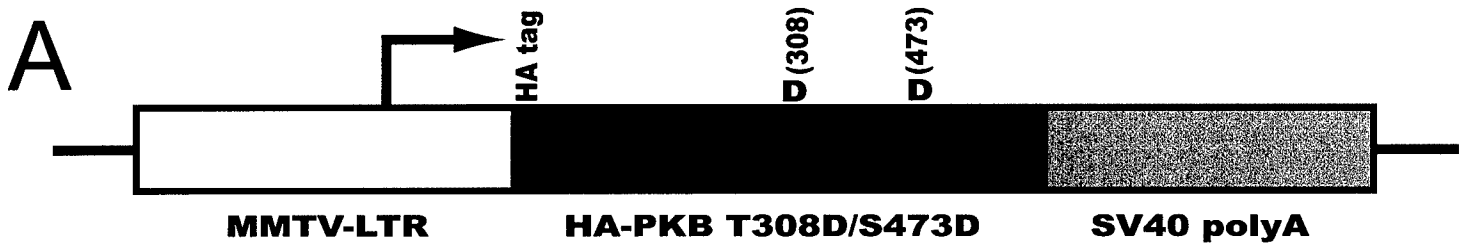
[c] Strain did not pass transgene.

Figure 1 - Transgene expression in MMTV/Akt and MMTV/PI3K transgenic mice

(A) Structure of the MMTV/Akt transgene. The Bluescript vector backbone is represented by a thin line on either side of the expression cassette, with the white region corresponding to the MMTV LTR derived from plasmid pAp, the black portion corresponding to the hemaglutinin tag, the dark grey region corresponding to the Akt (HAPKBT308D/S473D) cDNA with aspartate substitutions at amino acid positions 308 and 473, and the mid-grey region corresponding to the transcriptional processing sequences derived from the SV40 early transcription unit. The transcription start site is indicated by the arrow.

(B) Structure of the MMTV/PI3K transgene. As above, the Bluescript vector backbone is represented by a thin line on either side of the expression cassette, withe the white region corresponding to the tMMTV LTR derived from plasmid pA9 and the mid-grey region corresponding to the transcriptional processing sequences derived from the SV40 early transcription unit. The black portion corresponds to the ras-derived CAAX motif, the dark grey region represents the PI3K (p110CAAX) cDNA and the light-grey region represents the myc tag.

(C) Protein corresponding to the MMTV/Akt transgene in various organs of the Akt7 and Akt10 transgenic strains as assessed by Western blot against the HA tag (Babco HA-11). Tissues were derived from virgin females and males. Also shown are control Grb2 Western blots on identical protein samples. M.Gl., mammary gland; Epidid., epididymus; Sem.Ves., seminal vesicles.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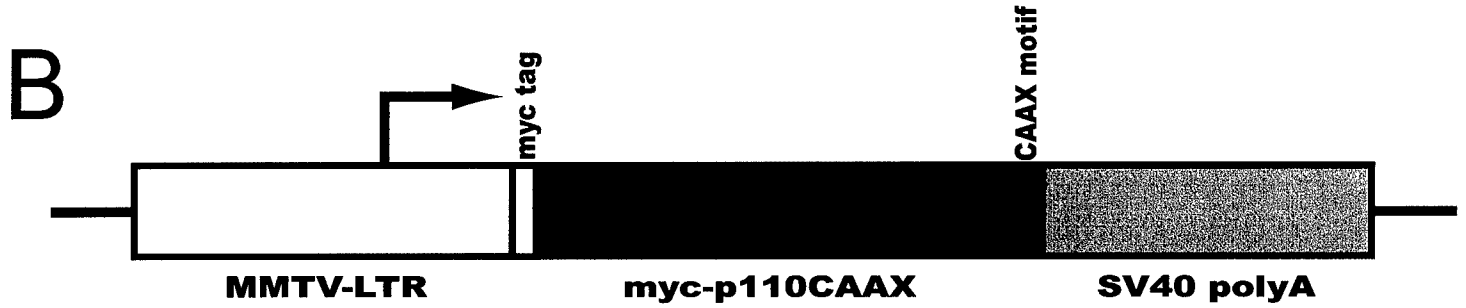
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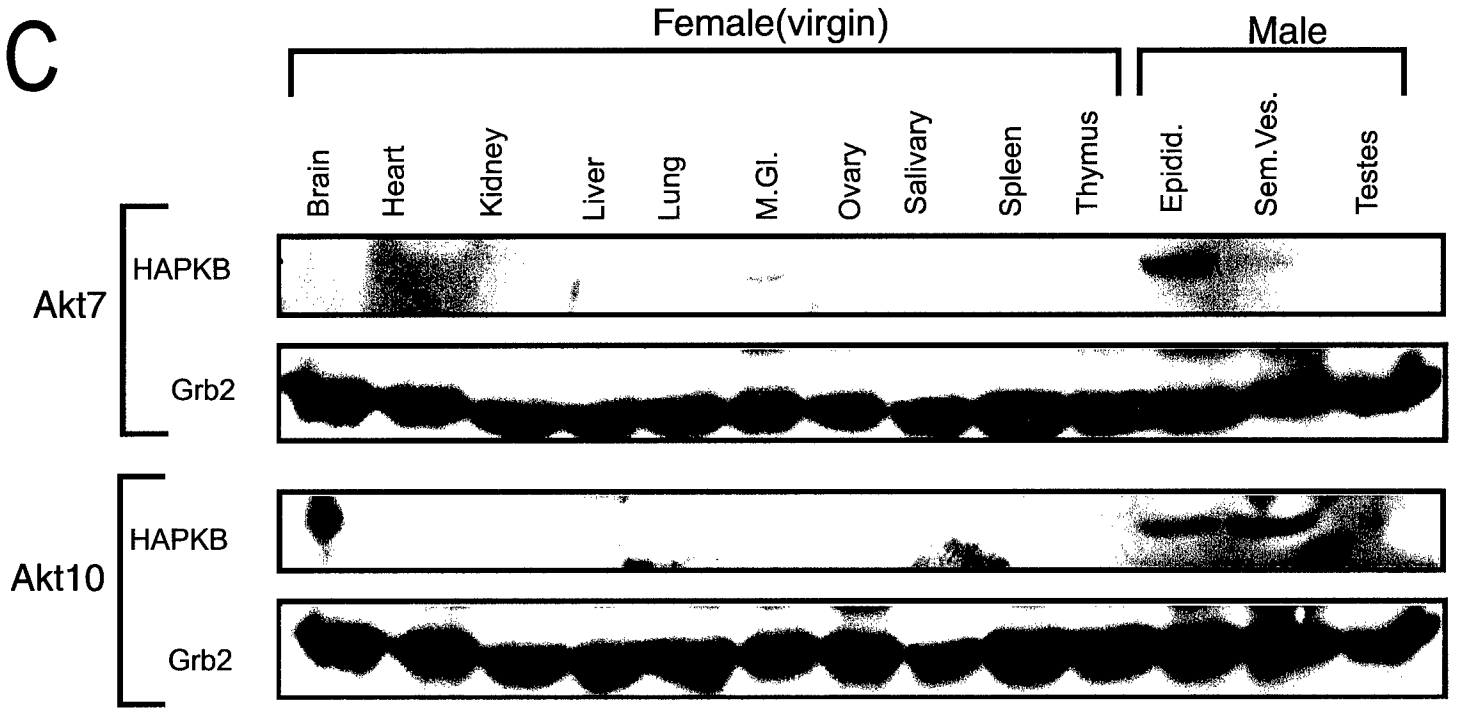
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Epidid.
Sem.Ves.
Testes
Akt7
HAPKB
Grb2
Akt10
HAPKB
Grb2

Figure 2 - Wholemount analysis of mammary glands from FVB/n, MMTV/Akt7, MMTV/MTY315/322F and MMTV/Akt7 X MTY315/322F transgenic animals.

Photomicrographs comparing the whole-mount (magnification, X10) appearances of virgin female FVB **(A)**, Akt7 **(B)**, MTY315/322F **(C)**, and Akt7 X MTY315/322F **(D)** mice 6.5 weeks after birth are shown.

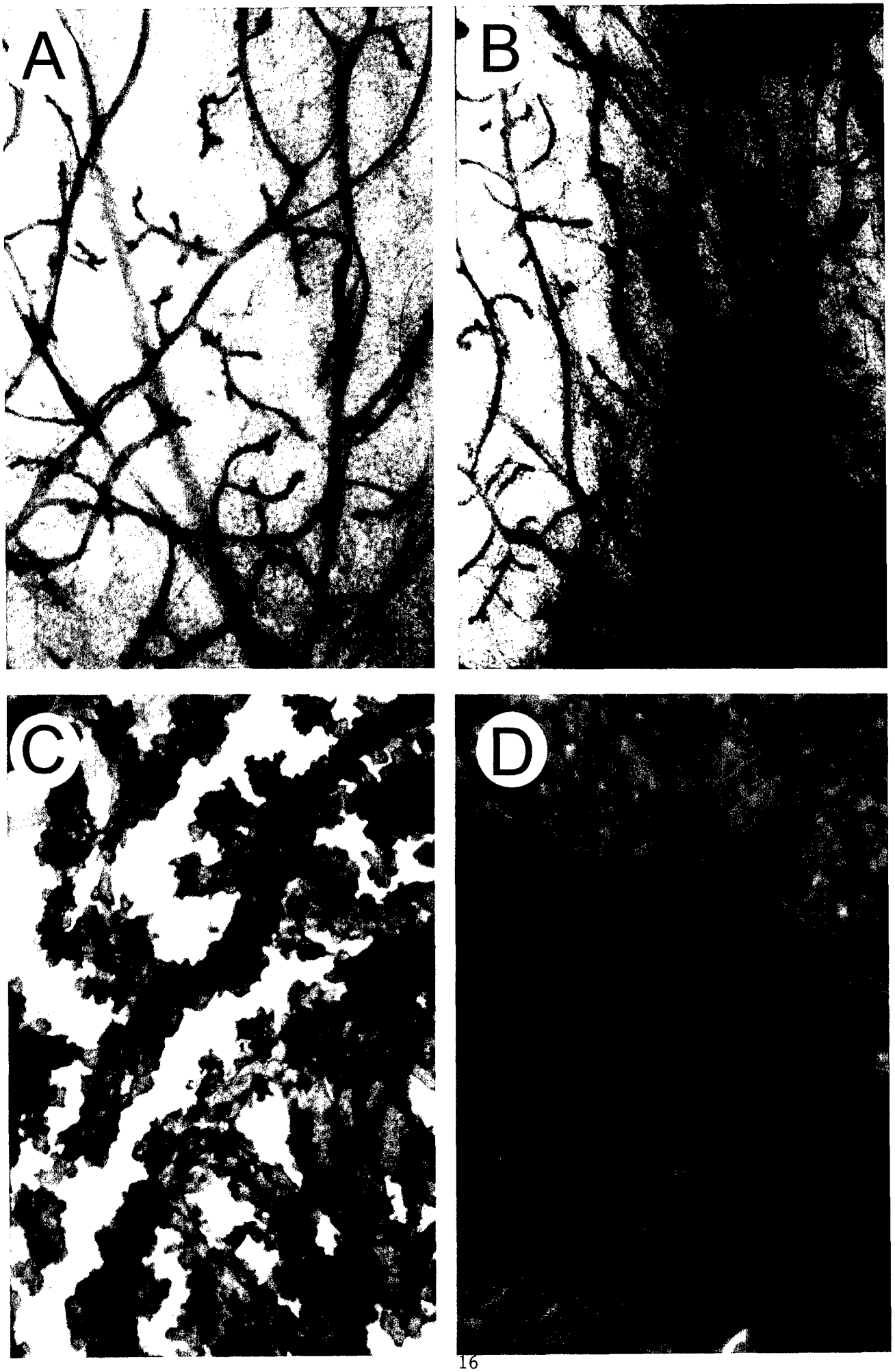

A
B
C
D

Figure 3 – Kinetics of mammary tumor occurrence in MMTV/MTY315/322F and MMTV/Akt7 X MTY315/322F transgenic animals.

Age indicated is that at which a mammary tumor is first palpable in each transgenic strain. The number of animals analyzed for each strain (n) and the median age at which tumors are palpable is also shown.

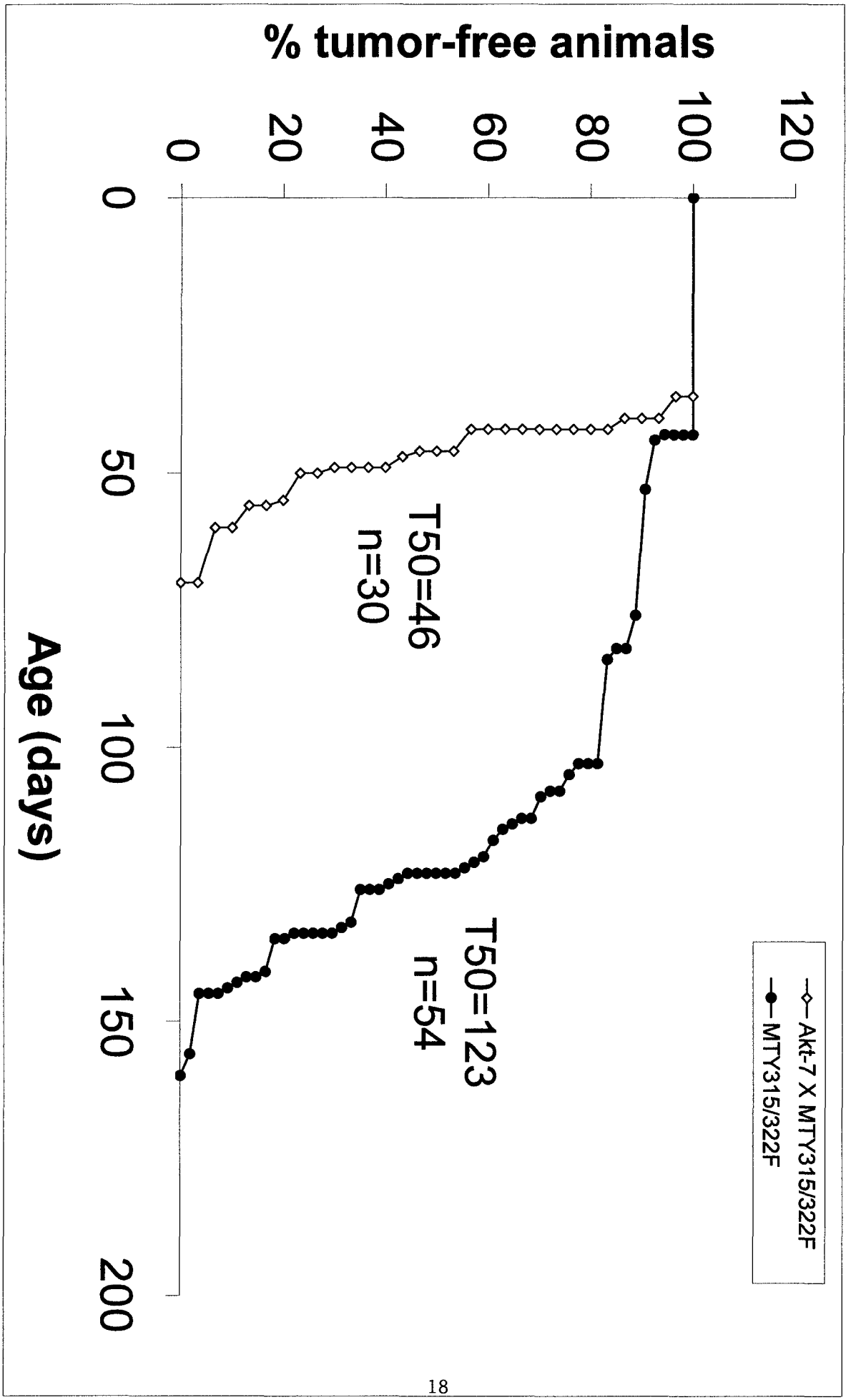
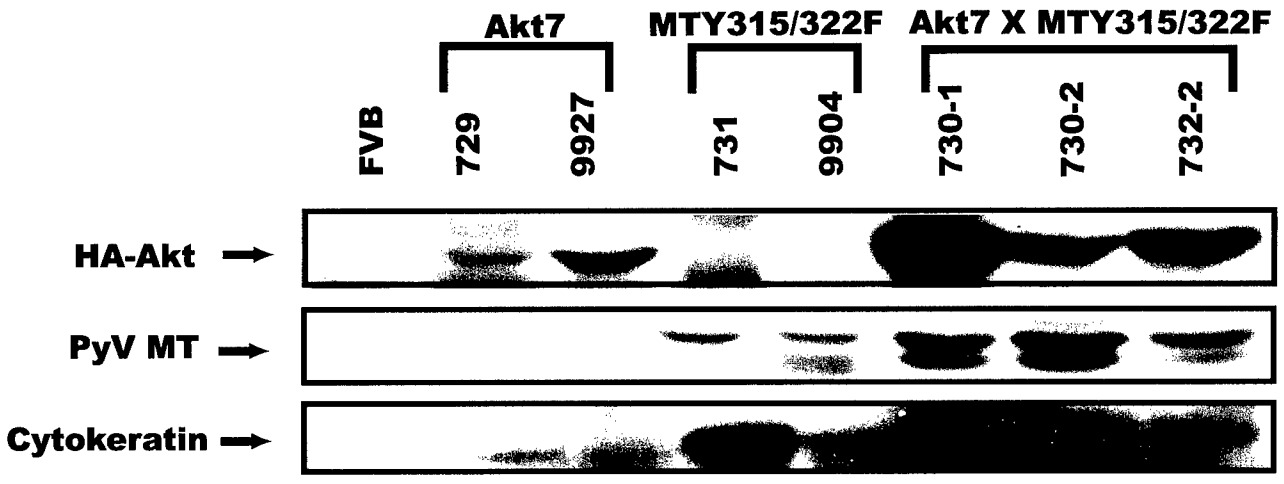

% tumor-free animals
120
100
80
60
40
20
0
0
50
100
150
200
Age (days)
T50=46
n=30
T50=123
n=54
Akt-7 X MTY315/322F
MTY315/322F

Figure 4 – Expression of HAPKB, PyVMT and cytokeratin in bi-transgenic Akt7 X MTY315/322F strains.

Immunoblot analyses of mammary tissue from the FVB, Akt7, MTY315/322F and Akt7 X MTY315/322F strains. Two-hundred and fifty micrograms of total protein lysate was pre-cleared in ProteinG-Sepharose and subjected to anti-HA immunoblot analysis with HA-11 monoclonal antibody (Babco). PyV MT was immunoprecipitated from two milligrams of total protein lysate with ~2 μg of Pab762 (courtesy Dr. S. Dilworth) and subjected to anti-MT immunoblot analysis with Pab701 (courtesy Dr. S. Dilworth). Anti-cytokeratin immunoblot analysis was carried out on 250 μg of total protein lysate using Troma-1 rat monoclonal antibody. Higher levels of Akt and MT may reflect higher epithelial cell content of tissue samples as revealed by anti-cytokeration analysis.


Akt7
MTY315/322F
Akt7 X MTY315/322F
FVB
729
9927
731
9904
730-1
730-2
732-2
HA-Akt
PyV MT
Cytokeratin

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Jun 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for the Accession Documents listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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